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Bezeichnung: Spontaneously beating neonatal rat heart myocyte culture-a model to characterise angiotensin II AT_1rezeptor autoantibodies in patients with preeclampsia

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Die angehefteten Stücke sind eine richtige und genaue Wiedergabe der ursprünglichen Unterlagen dieser Patentanmeldung.

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Der Präsident
Im Auftrag

Wallner

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SPONTANEOUSLY BEATING NEONATAL RAT HEART MYOCYTE CULTURE – A MODEL TO CHARACTERISE ANGIOTENSIN II AT_1 RECEPTOR AUTOANTIBODIES IN PATIENTS WITH PREECLAMPSIA

Spontaneously beating cultured neonatal rat cardiomyocytes are a very useful model to investigate action of autoantibodies. Research about β_1-adrenoceptor autoantibodies were already reported by Wallukat et al., 2001. This report is on AngiotensinII(AT_1) receptor autoantibodies in preeclamptic women. Preeclampsia is a disorder that is recognized due to increase blood pressure that can cause maternal and fetal mortality. Dechend et al., 2000 were fortunate to demonstrate the manifestation of agonistic antibodies to angiotensin AT_1 receptors prevalent in preeclamptic women. Activation of the AT_1 receptor by agonistic autoantibodies could explain many of the pathophysiological features of preeclampsia. Findings of Wallukat et al., 1999 revealed that immunoglobulin fractions and affinity purified antibodies from preeclamptic women could stimulate the AT_1 receptor of cultured cardiomyocytes. The addition of Losartan (1μM) decrease beats per minute. By neutralization experiments it could be demonstrated that IgG subclass 3 are responsible for increase of beating frequency.

According to these findings we developed an enzyme linked immunoassay for determination of Angiotensin II AT_1 receptor autoantibodies (anti-AT_1-AAB).

First, peptide solutions corresponding to the amino acid sequence of the second loop of the human AT_1 receptor (Sm 1986/1, 100µg/ml) were incubated with Anti-AT_1-AAB (1:1; v/v) for 1 hour at 4°C. Anti-AT_1-AAB's were prepared by ammonium sulphate precipitation from spoil fluids (blood and isotonic salt solution) during birth. These samples were more concentrated than pure serum probes. Second, this mixture was incubated with washed streptavidin coated magnetic particles for 1 hour at 4°C. Third, to remove IgG-peptide mixture magnetic particles were washed three times with wash buffer (20mM potassium-phosphate buffer, 0.15M NaCl, pH 7.5). Removal of solution or wash can be easily performed by using a magnetic concentrator (Dynal). Unspecific binding sites were blocked with 1% bovine serum albumin in wash buffer. Fourth, Magnetic particles were incubated with anti-human IgG 3 horse radish peroxidase labeled antibody solution (1: 200, 1 hour, room temperature). Fifth, the particles was treated with standardized ready to use solution TMB (Tetramethylbenzidine) for 30 min at room temperature in the dark. The color reactions (blue-green) were stopped with 0.1N HCl (yellow-orange). Optical densities were measured in a microplate reader (AnthosHTII) at 492 nm (reference filter 620 nm). Results are summarized in Table 1.

The same peptide of the human AT_1 receptor (Sm 1986/1) was used for purification of Anti-AT_1-AAB's. IgG solutions were mixed with peptide solution (100µg/ml, 1:1; v/v) and incubated one hour at 4°C. Three times washed Streptavidin coated magnetic particles (300 µl) were added. Particles were collected by magnetic concentrator. Supernatants were carefully removed and stored in ice. Magnetic particles were three times washed and eluted by 3 M Potassium thiocyanate solution for 15 min at room temperature. After magnetic concentration, solutions were carefully removed along with the first supernatant dialyzed against NaCl (0,9%) phosphate buffered solution. After 5 changes within 3 days protein content were determined by optical density (280 nm). The chronotropic effect of

supernatant and eluat were registrated on primary cultured neonatal rat cardiomyocytes (bioassay) with the computer imaging system (IMAGOQANT). Table 2 shows the reproducibility of the purification method. Six of six purified Anti-AT_1-AAB's showed the increase of beating frequency/min (> 24.4). In contrast , cultures treated with supernatant exert no or moderate changes of the beating frequency (<10.0).

The procedure of coimmunoprecipitation of AT_1 receptor was similar to the method of $ß_1$-Adrenoceptor (Wallukat 2001). The differences are: lysed membranes of transfected CHO-cells (Couchon 1997) were used for coimmunoprecipitation. Lysed membranes should be freshly prepared. Proteins were identified by an antibody directed against a peptide with the sequence of the N-terminal part of AT1-receptor produced in rabbit (N10, 1:100, Santa Cruz) and detected by Western blot and ECL system with anti rabbit IgG peroxidase conjugates (1:10.000,Sigma). Figure 1 shows results of Western blot. One band (molecular weight > 40.0 kDa) could be accurately detected using internal positive samples (lysed membranes of transfected CHO cells and human placenta tissue). In former experiments (Neichel, unpublished data) this band could be blocked by the peptide which were used for production of N10 antibodies. This band was missed in pure sepharose probes and supernatants of purification experiments.

Our results indicate the usefulness of the computer imaging system IMAGOQUANT to detect increase of beats/min by AT_1-AAB's in patients with preeclampsia. Enzyme linked immunoassay should be tested with sera of preeclampsia patients and healthy donors. Purified AT_1-AAB's can be used for further investigation of pathogenesis of preeclampsia.

TABLE 1

MEASUREMENT OF AT_1 AUTOANTIBODY BY ENZYME-LINKED IMMUNOASSAY

IgG	n	Optical density (OD, 492nm) Range
Healthy (Controls)	3	0.036 – 0.069
Preeclamptic woman		
positive	15	0.071 – 0.786
negative	4	0.021 – 0.069

TABLE 2

INFLUENCE OF SUPERNATANTS AND ELUATES OF MAGNETIC PARTICLES ON BEATING FREQUENCY OF CULTURED NEONATAL RAT CARDIOMYOCYTES

Patient/ Date of experiment	Probes	OD	µg/ml	Bioassay (Increase of beats /min) 1:100	1:50	1:20
D. 3-19-2	Supernat.	4.300	3071.4	6.0 ± 0.0	6.0 ± 0.0	10.0 ± 1.6
	Eluat	0.086	61.4	12.8 ± 1,6	27.6 ± 2,0	34.4 ± 1,2
D. 5-27-02	Supernat.	6.820	4871.4	-1.6 ± 0.8	4.0 ± 1.2	6.4 ± 1.2
	Eluat	0.033	23.6	12.1 ± 2.4	18.9 ± 0,5	24.5 ± 0.8
D. 6-03-02	Supernat.			3.3 ± 0.8	3.2 ± 0,8	4.7 ± 1.6
	Eluat	0.104	74.3	11.1 ± 1.2	15.2 ± 2,0	33.9 ± 2.0

± SD of the mean

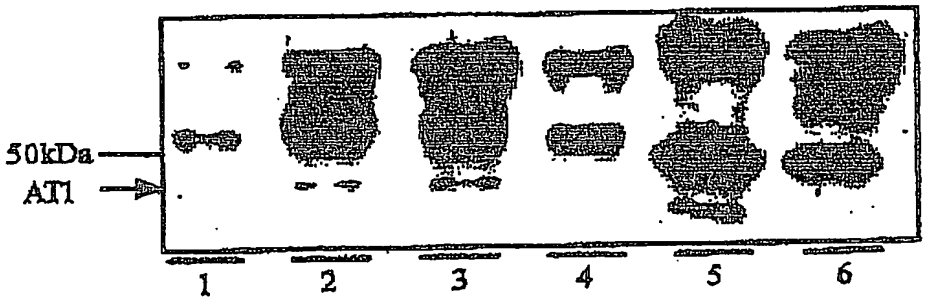

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Figure 1: Western blot of coimmunoprecipitation of Angiotensin AT_1 receptor

Lane1 ProteinA-sepharose; 2 preeclamptic patient D. without purification; 3 KSCN eluate; 4 supernatant ; 5 CHO lysed membranes; 6 placenta lysed tissue.

Tabelle 3: Autoantikörper gegen G-Protein gekoppelte Rezeptoren

Angaben zu den Epitopen und IgG Subklasse

Antikörper gegen Rez.	Erkrankung	Epitop	IgG Subklasse
Beta1-adren.	dilat. Kardiomyopathie	1. loop 2. loop	IgG3 u. IgG4 IgG1
Beta1-adren.	Chagas' Kardiomyopathie	2.loop	
Beta1-adren.	Myokarditis	1. loop 2. loop	IgG3 u. IgG4 IgG1
muskarin. M2	dilat. Kardiomyopathie	2. loop	IgG1
muscarin. M2	Chagas' Kardiomyopathie	2. loop	
Ang. II AT1	Präeklampsie	2. loop	IgG3
Ang. II AT1	humorale Nierenabstoßung	2. loop	IgG1 u. IgG3
Ang. II AT1	maligne Hypertonie	2. loop	IgG1 u. IgG3
Alpha1-adren.	essentielle Hypertonie	1. loop 2. loop	IgG1 u. IgG3 IgG2
Alpha1-adren.	refraktäre Hypertonie	1. loop 2. loop	IgG1 u. IgG3 IgG2
Alpha1-adren.	pulmonare Hypertonie		
Alpha1- adren.	Psoriasis	1. loop 2. loop	
PAR-1 u. PAR-2	Raynaud-Syndrom	2. loop	IgG1

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Claim

1. Method for determination of Angiotensin II AT_1 receptor autoantibodies comprising the steps

- incubation of a Anti-AT_1-ABB with a peptide solution corresponding to aminoacid sequence loop of a human AT1 receptor, whereby a mixture is obtained;
- incubation of the mixture with streptavidin coated magnetic particles,
- incubation of the magnetic particles with anti-human IgG peroxidase labeled antibody solution, whereby a solution is obtained,
- treatment of the solution with Tetramethylbenzidine, whereby a color reaction occurs,
- detection of the color reaction.